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Cancer Immunology Immunotherapy

Granulocyte-monocyte colony-stimulating-factor augments the interleukin-2-induced cytotoxic activity of human lymphocytes in the absence and presence of mouse or chimeric monoclonal antibodies (mAb 17-1A)

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Summary. Blood lymphocytes stimulated for 96 h with interleukin-2 (IL-2; 100 BRMP U/ml) (lymphokine-activated killer, LAK, cells) or granulocyte-monocyte colony-stimulating-factor (GM-CSF) (10 ng/ml) became cytotoxic for Daudi cells. IL-2 was significantly more effective than GM-CSF. Only IL-2-activated cells killed SW948 (a human colorectal carcinoma cell line) while GM-CSF-stimulated cell did not. GM-CSF and IL-2 acted synergistically in a dose-dependent fashion for induction of a highly effective cytotoxic cell population (IL-2/GM-CSF cells). Il-2/GM-CSF cells were statistically significantly more effective than LAK cells in lysing Daudi cells and SW948 ($P < 0.05$). The enhancing effect was most pronounced during the first 48–96 h of activation. Incubation periods longer than 192 h did not contribute to augmented cytotoxicity. The combination of IL-2 and GM-CSF significantly increased the number of $CD25^+$ cells compared to IL-2 and GM-CSF alone. Furthermore, IL-2/GM-CSF cells were significantly more effective in antibody-dependent cellular cytotoxicity assays (SW948 + mAb 17-1A) than LAK cells. The chimeric mAb 17-1A was significantly more effective in tumor cell lysis than the mouse mAb. Thus, combination of various biological therapeutics might be a way to enhance their antitumoral effects.

Introduction

Granulocyte-monocyte colony-stimulating-factor (GM-CSF) regulates proliferation and differentiation of granulocytes and monocytes/macrophages [3, 5, 18, 19]. It inhibits neutrophil migration and phagocytosis, stimulates macrophage and granulocyte cytotoxicity and augments antibody-mediated tumoricidal activity in vitro [4, 7]. We have recently shown that GM-CSF can increase not only monocyte but also lymphocyte cytolytic activity in an 18-h ADCC (antibody-dependent cellular cytotoxicity) assay mediated by mouse or chimeric mAb (monoclonal antibody) 17-1A against SW948, a human colorectal carcinoma cell line [15]. The ADCC activity in this system could also be significantly enhanced by preactivation of the effector cells with IL-2 [16], and further increased by simultaneous addition of tumor necrosis factor α (TNFα) [28]. Furthermore, GM-CSF alone or in combination with IL-2 increased proliferation and the mixed lymphocyte culture activity of blood lymphocytes and T cells [24].

The arsenal of biological therapeutics for the treatment of cancer patients includes mAb, cytokines and activated cytotoxic cells. Each of these components alone might induce tumor regression [1, 6, 8, 17, 21, 29]. Using naked antibodies alone, the main effector function seems to be ADCC mediated by monocytes/macrophages and natural killer (NK) cells [15, 16]. The clinical effect is, however, low and if mAb therapy is to become widely clinically applicable the therapeutic effectiveness must be increased. This might be achieved by augmentation of the cytolytic capability of the effector cells. A possible scenario in the future using biological therapeutics may be the combination of various agents with different activities on the immune system.

The aim of the present study was to analyze whether the cytolytic capacity in vitro in ADCC could be enhanced by activation of the effector cells with GM-CSF and IL-2 in combination. These two cytokines are in clinical use today. Such preclinical information should be of great value for the design of therapeutic protocols.

Materials and methods

Human effector cells. Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood from healthy adult donors, and separated by centrifugation on a Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) gradient (density: 1.077 g/ml) [2]. The cells were washed twice in phosphate-buffered saline PBS and incubated with iron powder for 30 min at 37° C. Phagocytic cells were removed by a magnet [13].

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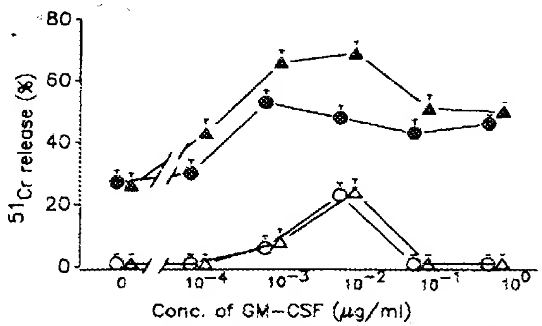

Fig. 1. Lymphocytes were cultured for 48 h (○, ●) and 96 h (△, ▲) in the absence of (○, △) or in the presence of (●, ▲) 100 BRMP U/ml IL-2 and different concentrations of GM-CSF. Target cells: Daudi. The percentage (mean ± SE) ^{51}Cr release of triplicates in a 4-h assay is shown at a 15 : 1 E/T ratio (one representative experiment)

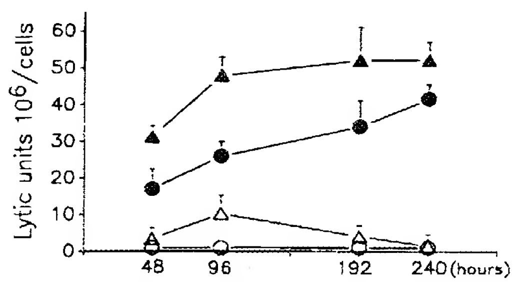

Fig. 2. Lymphocytes were cultured for various time periods with GM-CSF (10 ng/ml) and IL-2 (100 BRMP U/ml) (▲); IL-2 (100 BRMP U/ml) alone (●); GM-CSF (10 ng/ml) alone (△) and medium only (○). Cytotoxicity (LU/10^6 cells) (mean ± SE) ($n = 5$) was tested against Daudi cells. Significance levels (paired t-test): ▲ vs ● $P < 0.05$ at all incubation times; △ vs ○ $P < 0.01$ at 48 h, 96 h and 192 h but not significant at 240 h

Cytokines. Recombinant human IL-2 was kindly supplied by Du Pont de Nemours & Co Inc., Wilmington, Del, USA. The specific activity was 2.6×10^6 BRMP U/mg protein with 4.59 endotoxin U/ml as determined by *Limulus* amoeloocyte lysate assay (M. A. Bioproducts, Walkersville, USA). Recombinant human granulocyte-macrophage colony-stimulating factors (GM-CSF) (biological activity F-5 $\times 10^7$ colony-forming units/mg proteins) was supplied by Behringwerke AG, D-3550, Marburg, FRG.

IL-2 and GM-CSF activation of cells. PBL were cultured in AIM V (Gibco/BRL, Grand Island, NY, USA) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 20 µg/ml gentamycin, 100 µg/ml streptomycin, 100 U/ml penicillin. Various concentrations of IL-2 and/or GM-CSF were added as described. Effector cells were preincubated for various time periods. The cells (3×10^6/ml) were cultured in humidified air with 5% CO_2 at 37°C. The effector cells were washed three times in phosphate-buffered saline before use in the cytotoxic assay.

Cell subpopulations. PBL were analyzed for subpopulations by indirect immunofluorescence using an absorbed goat anti-mouse IgG antibody or rabbit anti-mouse IgG F(ab')$_2$ fragments in the second step (Becton Dickinson, Mountain View, Calif, USA; DAKO A/S, Copenhagen, Denmark). Before staining, the cells were incubated at 37°C for 30 min and washed twice in Hanks/TRIS solution. The following antibodies were used: Leu4 (CD3, PAN T antigen), Leu11 (CD16, NK cells/neutrophils), anti-Tac (CD25, Il-2R), LeuM1 (CD15, monocytes/macrophages), LeuM5 (CD11c, monocytes/macrophages), B1 (CD20, B cells). Samples of 10^4 cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, Calif, USA) at 480 nm with a flow rate of <300 cells/s.

Monoclonal antibodies used in ADCC. The mouse mAb 17-1A (IgG$_{2A}$-K) and the chimeric (mouse × human) mAb 17-1A (IgG$_1$-K) against the tumor-associated antigen (CO17-1A) were used [14, 26]. Native and chimeric mAb 17-1A have similar antigen specificity and affinity as described in detail by Shaw et al. [25]. The mouse mAb F$_i$ (IgG$_{2A}$-K directed against the variable part of the T$_i$ cell receptor on a T chronic lymphatic leukemia [10]) and normal mouse IgG were used as controls.

Cell lines. Target cells used were: Daudi, a B cell line [11]; SW948, a colorectal carcinoma cell line expressing the antigen CO17-1A [9].

Cytotoxicity tests. The cytotoxic activity of activated cells was determined in a 4-h ^{51}Cr-release assay. After trypsinization of the target cells and testing for viability (trypan blue) the cells were labelled with 2.8 MBq sodium [^{51}Cr]chromate/ml cells (sp. act. 9–18.5 GBq/mg chromium, Amersham International, England) at 37°C for 1 h. After washing three times in full medium (Leibovotz medium 15, Gibco Ltd, Scotland, containing 10% fetal calf serum, antibiotics and 2 mmol L-glutamine) the cells were added to round-bottom wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark) and effector cells were added to give effector-to-target cell ratios of 30, 15, 8. Antibodies were added at the concentrations required by the experimental design. Supernatants were harvested by the Skatron Titer Tec System (Skatron A/S, Lierbyen, Norway) and counted in a gamma counter. Maximum isotope release was measured by incubation of the target cells with 5% Triton-X (Merk, Darmstadt, FRG). Spontaneous release was determined by incubation of the ^{51}Cr-loaded target cells with medium alone. The percentage specific lysis was calculated by the formula:

$$\text{Lysis (\%)} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Data are shown as either the mean percentage lysis of triplicate values or as lytic units (LU)/10^6 cells; 1 LU being defined as the number of PBL required to obtain 30% specific lysis according to the method of Pross et al. [22].

Statistics. Analyses of differences between means were done by the paired Student's t-test and the non-parametric Wilcoxon's test.

Results

Freshly isolated blood mononuclear cells contained >98% lymphocytes; 78 ± 6% were CD3+, 5 ± 4% CD20+ and 18 ± 4% CD16+ ($n = 5$).

Dose requirements for the generation of cytotoxic cells using the combination of GM-CSF and IL-2

A dose of 100 BRMP U/ml IL-2 alone induced optimal lymphokine-activated killer (LAK) cell activity of blood lymphocytes cultured for 48–96 h. Higher concentrations did not significantly increase the lytic activity [28] (data not shown). GM-CSF at 10 ng/ml also induced a cytotoxic cell population when cultured for 48–96 h (Fig. 1). GM-CSF had a narrow range of activity (1–10 ng/ml).

Lymphocytes were also cultured for 48–96 h with a constant concentration of IL-2 (100 BRMP U/nml) and increasing concentrations of GM-CSF (Fig. 1). The simultaneous addition of 1–10 ng/ml GM-CSF and 100 BRMP

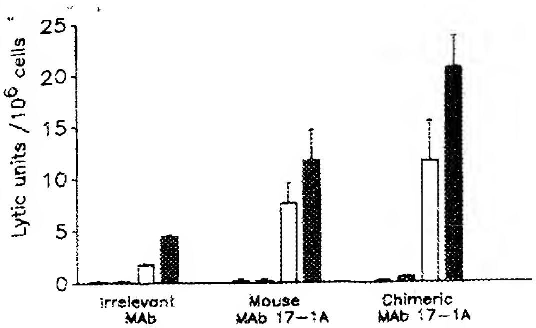

Fig. 3. Cytotoxicity (LU/10^6 cells) (mean $\pm$ SE) ($n = 3$) of lymphocytes activated for 48 h with IL-2/GM-CSF (■), IL-2 (□), GM-CSF (▩) and medium only (▩) against SW948 in the presence of mouse mAb 17-1A (1 µg/ml), chimeric mAb 17-1A (1 µg/ml) and an irrelevant mAb F1 (1 µg/ml). The concentration of IL-2 was 100 BRMP U/ml and of GM-CSF 10 µg/ml. 4-h ^{51}Cr-release assay. The differences in activity between IL-2 and IL-2/GM-CSF cells for each mAb as well as between the different mAB were statistically significant ($P < 0.05$)

Table 1. Cytotoxic activity against Daudi cells after activation with granulocyte-monophage colony-stimulating-factor (GM-CSF; 10 ng/ml) and various concentrations of interleukin-2 (IL-2) for 96 h and 192 h respectively (two representative experiments)

Combination of cytokines	Concentration of IL-2 (BRMP U/ml)	Donor	Cytotoxic activity (LU/10^6 cells) after culture for: 96 h	192 h
Medium only	–	1	0	N. D.[a]
	–	2	0	N. D.[a]
GM-CSF only	–	1	2	<1
	–	2	3	<1
IL-2 only	10	1	3	19
	10	2	4	N. D.
IL-2/GM-CSF	10	1	16	30
	10	2	20	N. D.
IL-2 only	100	1	18	36
	100	2	25	41
IL-2/GM-CSF	100	1	35	56
	100	2	40	62

[a] ^{51}Cr-release test could not be performed at this time of the culture period as all cells were dead

U/ml IL-2 to the cells for 96 h significantly enhanced their lytic capacity.

Lymphocytes were also cultured with a constant dose of GM-CSF (10 ng/ml) and two doses of IL-2 (10 and 100 BRMP U/ml) for 96 h and 192 h (Table 1). GM-CSF augmented the cytotoxic capacity in presence of 10 BRMP U/ml as well as 100 BRMP U/ml IL-2. The most effective cell population was obtained by culturing the lymphocytes for 192 h with 100 BRMP U/ml IL-2 and 10 ng/ml GM-CSF.

Time kinetics for induction of cytotoxic cells by GM-CSF and IL-2

GM-CSF alone induced optimal lytic activity after preactivation for 96 h (Fig. 2). A decreased lytic capability was noted when incubated for longer times in spite of replacement with fresh medium and the cytokine. Stimulation with IL-2 alone continuously augmented the lytic capacity (fresh medium and IL-2 were added at 96 h). A combination of GM-CSF and IL-2 induced the strongest cytotoxic cell population. The maximum level was reached at 192 h, while the increment was most obvious at 96 h of incubation.

Expression of IL-2 receptors (CD25) on activated cells

During the 96-h culture period there was a significant increase in the fraction of CD25$^+$ lymphocytes after stimulation with IL-2 alone but not with GM-CSF, compared to the original population and unstimulated cells (Table 2). However, stimulation with GM-CSF and IL-2 simultaneously significantly increased the proportion of CD25$^+$ cells compared to IL-2-stimulated, GM-CSF-activated and unstimulated cells respectively. When culturing for 192 h, the increase in CD25$^+$ cells was still more marked when the combination of GM-CSF and IL-2 was used and significantly higher compared to the cell population stimulated with IL-2 alone. Furthermore, the numbers of CD25$^+$ cells were significantly higher at 192 h compared to 96 h. The numbers of CD3$^+$, CD4$^+$, CD8$^+$ and CD16$^+$ cells did not differ significantly between the various culture conditions (data not shown).

Table 2. CD25$^+$ cells after activation with different cytokines

Activation time (h)	CD25$^+$ cells (%) Medium	GM-CSF, 10 ng/ml	IL-2, 100 BRMP U/ml	GM-CSF/IL-2
0 ($n = 8$)	$2.9 \pm 0.9^{1*}$	–	–	–
96 ($n = 8$)	2.2 ± 0.4^{2}	2.3 ± 0.3^{3}	$5.8 \pm 0.9^{1,2,3,4,8}$	$8.6 \pm 1.8^{1,2,3,4}$
192 ($n = 5$)	2.2 ± 0.4^{5}	2.7 ± 0.7^{6}	$12.2 \pm 2.6^{1,5,6,7,8}$	$16.6 \pm 4.8^{1,5,6,7,8}$

* Superscripts indicate between which values the comparison was made. In all comparsons the p values were <0.05 using both paired Student's t-test and Wilcoxon's non-parametric test

IL-2/GM-CSF cells in ADCC

Lymphocytes were preactivated for 48 h with GM-CSF, IL-2 and GM-CSF + IL-2 respectively, before assay in ADCC mediated by the mouse or the chimeric mAb 17-1A against the human colorectal carcinoma cell line SW948. Unstimulated lymphocytes as well as lymphocytes activated with GM-CSF for 48 h did not lyse the tumor cells in the absence of mAb (irrelevant mAb), while IL-2-activated and IL-2/GM-CSF-activated cells did (Fig. 3). Addition of mouse mAb 17-1A significantly enhanced the lytic capability, which was even more marked when the chimeric mAb was added. Il-2/GM-CSF cells were consistently significantly more effective than IL-2-activated cells.

Discussion

Lymphocytes stimulated by IL-2 or GM-CSF for only 48–96 h generated cytotoxic cells against Daudi cells but the stimulatory effect of GM-CSF was weak compared to that of IL-2. Only IL-2-activated but not GM-CSF-activated cells lysed SW948 tumor cells. Preactivation of the effector cells by adding IL-2 and GM-CSF simultaneously resulted in a far more effective cell population compared to IL-2 alone. This was the case not only for spontaneous cytotoxicity against Daudi cells and SW948 but also for ADCC. These results may be in line with those of Santoli et al. [24] showing that GM-CSF acted synergistically with IL-2 on the proliferation of unfractionated lymphocytes and T cells. The activity was most marked in the unfractionated cell population.

The explanation for the increased lytic capability is not clear. In the cell population stimulated with IL-2 and GM-CSF a higher fraction expressed CD25, which might render more cells susceptible to IL-2 stimulation and consequently a larger fraction of activated cells might be available for lytic action. The increased cytotoxicity might also be an effect of an augmented superoxide production [23, 27]. Furthermore, GM-CSF has been shown to increase the expression of FcRII on myeloid leukemic cells, neutrophils, eosinophils and monocytes [12]. FcRII seems to be an important receptor for ligation of the Fc part of antibodies and thus for antibody-mediated cytotoxicity [20]. In the present study, FcRII was not analyzed, which may be of importance for gaining further insight into mechanisms responsible for the increased cytotoxicity seen after stimulation with GM-CSF. However, FcRIII (CD16) did not change significantly during the culture period (data not shown). Moreover, in our system the chimeric mAb was consistently significantly more effective than the mouse mAb, which has also been described earlier in other in vitro systems [15, 16, 25, 28]. If ADCC is an important mechanism for killing tumor cells in vivo, chimeric mAb should be of preference.

A tenfold lower dose of IL-2 was required when combined with GM-CSF as compared to IL-2 alone to generate a cytotoxic cell population with the same lytic activity (Table 1). These findings are similar to those describing a synergistic effect of low concentrations of IL-2 on the amplification of IL-2-driven T-cell proliferation with IL-3 and GM-CSF [24] and on the increase of IL-2-stimulated cell cytotoxicity with TNFα [unpublished results]. Thus, in the clinical situation low doses of IL-2 combined with GM-CSF might give a tumoricidal activity similar to that induced by high doses of IL-2 alone but probably with fewer side-effects.

Treatment of cancer patients with bolus or constant infusion of GM-CSF alone for 14 days increased the number of monocytes as well as their ADCC activity and the capacity to secrete TNFα and interferon γ [28]. Furthermore, GM-CSF alone was shown to induce some tumor regression in a few patients with solid tumors receiving GM-CSF in a phase I study (Morstyn, personal communication). Thus, combination of mAb, GM-CSF and IL-2 might be a way to increase the antitumoral effects of these biological therapeutics in cancer patients.

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